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# Physical and Genetic Map of the *Lactococcus lactis* subsp. *cremoris* MG1363 Chromosome: Comparison with That of *Lactococcus lactis* subsp. *lactis* IL 1403 Reveals a Large Genome Inversion†

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**A physical and genetic map of the chromosome of the *Lactococcus lactis* subsp. *cremoris* reference strain MG1363 was established. The physical map was constructed for *Not*I, *Apa*I, and *Sma*I enzymes by using a strategy that combines creation of new rare restriction sites by the random-integration vector pRL1 and ordering of restriction fragments by indirect end-labeling experiments. The MG1363 chromosome appeared to be circular and 2,560 kb long. Seventy-seven chromosomal markers were located on the physical map by hybridization experiments. Integration via homologous recombination of pRC1-derived plasmids allowed a more precise location of some lactococcal genes and determination of their orientation on the chromosome. The MG1363 chromosome contains six rRNA operons; five are clustered within 15% of the chromosome and transcribed in the same direction. Comparison of the *L. lactis* subsp. *cremoris* MG1363 physical map with those of the two *L. lactis* subsp. *lactis* strains IL1403 and DL11 revealed a high degree of restriction polymorphism. At the genetic organization level, despite an overall conservation of gene organization, strain MG1363 presents a large inversion of half of the genome in the region containing the rRNA operons.**

*Lactococcus lactis* is a gram-positive mesophilic bacterium that is used extensively in starter cultures in the manufacture of dairy products. There has been much research over the last decade to investigate its genetics. Because of the industrial importance of plasmids and bacteriophages, initial genetic studies tended to focus on these extrachromosomal elements. More recently, there has been an increased interest in chromosomal genes, and more than 80 markers have now been characterized. In contrast, little information is available about the structure and the organization of the chromosome of *Lactococcus* strains, mainly because, as for the majority of bacteria, study of the genome of lactococci cannot be done by the classical genetic mapping systems such as interrupted conjugation, transformation, and transduction. The physical and genetic map of the chromosome of various lactococci would be valuable for studying the organization and phylogeny of the lactococcal genome and to indicate the relatedness between the different species and subspecies of the lactococci. Pulsed-field gel electrophoresis (PFGE) is a powerful tool for genome characterization and has led to the construction of the physical map of more than 80 bacterial chromosomes.

PFGE was used in several studies of the genome of *L. lactis* to estimate the genome size and for genome fingerprinting (54, 58, 84), to analyze plasmid stability (92), and to study the integration sites of transposon Tn*917* derivatives (41). However, only two complete restriction maps of *L. lactis* have been published. The first map constructed was a *Not*I and *Sma*I physical map of the *L. lactis* subsp. *lactis* DL11 strain (86), a proteinase-negative ($Prt^-$) derivative of the nisin-producing strain ATCC 11454, and the second was a combined physical and genetic map of the commonly used laboratory strain *L. lactis* subsp. *lactis* IL1403 (49), a plasmid-free derivative of IL594 (12).

A prime candidate for chromosome mapping is the lactococcal reference strain *L. lactis* subsp. *cremoris* MG1363 (29). MG1363 is a plasmid-free derivative of strain NCDO 712, which is the ancestor (16) of the well-studied group of lactococcal strains NCDO 763 (ML3), NCDO 505, NCDO 2031, C2, and all strains derived therefrom, including the plasmid-free strain LM2301 (65). These strains constitute the most extensively studied group of lactococcal strains in terms of genetics and molecular biology.

According to phenotypic characteristics, the NCDO 712 group was formerly classified as *L. lactis* subsp. *lactis*, but DNA-DNA reassociation studies indicated that this group was not representative of the majority of the subspecies *lactis* (42). Recent data from 16S rRNA sequencing (43, 80) and Southern analysis with probes specific for the subspecies *lactis* (34) has demonstrated that the NCDO 712 group belongs to the subspecies *cremoris.*

We present the construction of the *Apa*I, *Sma*I, *Not*I, and I-*Ceu*I physical map of the *L. lactis* subsp. *cremoris* MG1363 chromosome and the localization of various chromosomal genes. Comparison of this map with the genome map of the two *L. lactis* subsp. *lactis* strains DL11 and IL1403 gives information about the chromosomal genetic organization at the subspecies level.

(A preliminary account of part of this work was presented previously [52].)

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *L. lactis* subsp. *cremoris* MG1363 (29) and *L. lactis* subsp. *lactis* IL1403 (12) were grown at 30°C in GM17 (M17 broth [85] supplemented with 0.4% glucose). *Escherichia coli* TG1 [*supE hsd*Δ*5 thi* Δ(*lac-*

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† This article is dedicated to Isabelle Kremper.

TABLE 1. Sizes of restriction fragments assigned to the MG1363 chromosome

| Enzyme | Fragment | Size (kb) | Enzyme | Fragment | Size (kb) | Enzyme | Fragment | Size (kb) |
|---|---|---|---|---|---|---|---|---|
| *Apa*I | Ap1A | 205 | | Ap19B | 33 | | Sm8 | 105 |
| | Ap1B | 205 | | Ap19C | 33 | | Sm9 | 58 |
| | Ap2 | 175 | | Ap20 | 28 | | Sm10 | 49 |
| | Ap3 | 160 | | Ap21 | 24 | | Sm11A | 42 |
| | Ap4 | 145 | | Ap22 | 23 | | Sm11B | 42 |
| | Ap5 | 115 | | Ap23 | 22 | | Sm11C | 42 |
| | Ap6 | 100 | | Ap24A | 20 | | Sm12 | 38 |
| | Ap7 | 83 | | Ap24B | 20 | | Sm13 | 31 |
| | Ap8 | 81 | | Ap24C | 20 | | Sm14A | 22 |
| | Ap9 | 78 | | Ap25 | 17 | | Sm14B | 22 |
| | Ap10A | 75 | | Ap26 | 16 | | Sm14C | 22 |
| | Ap10B | 75 | | Ap27A | 13 | | Sm15 | 18 |
| | Ap10C | 75 | | Ap27B | 13 | | Sm16 | 16 |
| | Ap11 | 69 | | Ap28 | 12 | | Sm17 | 11 |
| | Ap12A | 65 | | Ap29 | 8 | | Sm18 | 4 |
| | Ap12B | 65 | | Ap30 | 4.5 | | Sm19 | 3 |
| | Ap13 | 59 | | Ap31 | 3 | | | |
| | Ap14A | 49 | | | | *Not*I | No1 | 2,215[a] |
| | Ap14B | 49 | *Sma*I | Sm1 | 610 | | No2 | 230 |
| | Ap14C | 49 | | Sm2 | 325 | | No3 | 115 |
| | Ap14D | 49 | | Sm3 | 290 | | | |
| | Ap15 | 45 | | Sm4 | 220 | I-*Ceu*I | Ce1 (*rrnE*/*F*) | 1,533[a] |
| | Ap16 | 42 | | Sm5A | 180 | | Ce2 (*rrnA*/*F*) | 530 |
| | Ap17 | 38 | | Sm5B | 180 | | Ce3 (*rrnD*/*E*) | 240 |
| | Ap18 | 36 | | Sm6 | 130 | | Ce4 (*rrnB*/*C*) | 80 |
| | Ap19A | 33 | | Sm7 | 125 | | Ce5 (*rrnC*/*D*) | 55 |
| | | | | | | | Ce6 (*rrnA*/*B*) | 22 |

[a] The sizes of the largest *Not*I and I-*Ceu*I fragments were deduced from the size of the entire chromosome.

*proAB*) (F′ *traD36 proAB*$^+$ *lacI*$^q$ *lacZ*Δ*M15*)] was grown at 37°C with shaking in LB broth. When required, erythromycin was used at 150 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*.

**DNA preparation and digestion.** Plasmid isolation, restriction digestion, ligation, and transformation of *E. coli* were performed as described by Maniatis et al. (61). *Lactococcus* strains were transformed by electroporation (74) except that the cells were grown in GM17 supplemented with 2% glycine. Restriction enzymes and T4 DNA ligase were used as recommended by the suppliers. DNA restriction fragments were purified from agarose gel with the Prep-A-Gene DNA purification kit (Bio-Rad).

Lactococcal genomic DNA was purified and digested by slight modifications of the procedure of McClelland et al. (64). Cells were grown in 5 ml of GM17 containing 100 mM D,L-threonine to an optical density at 600 nm of 1 and centrifuged at room temperature (5 min, 3,000 rpm). The pellet was resuspended in 2.5 ml of TEE buffer (10 mM Tris-HCl [pH 9], 100 mM EDTA, 10 mM EGTA [ethylene glycol tetraacetic acid]), mixed with 2.5 ml of 2% low-geling-point agarose (SeaPlaque; FMC Corp.) prepared in TEE. The melted mixture was transferred to a mold and incubated at 4°C for 15 min to allow polymerization of agarose plugs. Plugs were then treated for 2 h at 37°C in 5 volumes of lysozyme buffer (5 mg of lysozyme per ml and 0.05% Sarkosyl in TEE) and overnight at 55°C in 5 volumes of proteinase K buffer (1 mg of proteinase K per ml and 1% sodium dodecyl sulfate [SDS] in TEE). Agarose plugs were washed for 1 h at room temperature in 50 volumes of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) in the presence of 0.1 mM phenylmethylsulfonyl fluoride and twice for 1 h each in 50 volumes of TE and stored at 4°C.

Total digestion of purified DNA was performed as recommended (64), except that agarose plugs were treated for 1 h on ice with 1 ml of TE 10/100 (10 mM Tris-HCl [pH 7.5], 100 mM EDTA) after restriction digestion. This treatment substantially enhances the resolution of restriction fragments of <20 kb in PFGE.

Embedded DNA was partially digested with *Apa*I or *Sma*I as already described (49).

**Electrophoresis.** Classical agarose gels were made up at 1% with 0.1 M TBE (1 M TBE is 1 M Tris base, 1 M boric acid, and 20 mM EDTA). PFGE was performed in a CHEF system (Pulsaphor Plus, LKB-Pharmacia) with 0.05 M TBE, as described previously (53). Standard size markers were phage λ DNA concatemers, obtained by the procedure of Waterbury and Lane (94), Megabase II DNA standards (Gibco-BRL), and a 1-kb DNA ladder (Gibco-BRL).

**Southern hybridization analysis of dried gels.** PFGE gels were treated with 0.5 N NaOH–0.15 M NaCl for 30 min and with 0.5 M Tris-HCl (pH 7.5)–0.15 M NaCl for another 30 min and dried at 60°C for 1 h with a Slab Dryer 483 (Bio-Rad). Dried gels were stored between two sheets of 3mm Whatman at room temperature until use. For high-stringency hybridizations, dried gels were hybridized overnight at 42°C in hybridization buffer (50% formamide, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 mM EDTA, 0.1% SDS, 2.5% skim milk) in a hybridization oven (Appligene). The gels were washed twice in 2× SSC–0.1% SDS for 20 min at room temperature and twice in 0.1× SSC–0.1% SDS for 20 min at 60°C. Low-stringency conditions were 30% formamide–6× SSC–5 mM EDTA–0.1% SDS–2.5% skim milk for the hybridization buffer at 42°C, with two washes in 2× SSC–0.1% SDS for 20 min at room temperature and two in 2× SSC–0.1% SDS for 20 min at 55°C. DNA probes were labeled with [α-$^{32}$P]dATP by using the Megaprime DNA labeling system (Amersham).

## RESULTS

**Identification of *Apa*I, *Sma*I, and *Not*I restriction fragments and genome size of MG1363 chromosome.** Because of the low G+C content of *Lactococcus*, *Streptococcus*, and *Enterococcus* strains (35 to 40%), the most suitable restriction endonucleases for genome mapping are the 6-bp recognition enzymes *Apa*I (GGGCCC) and *Sma*I (CCCGGG) and the 8-bp recognition enzyme *Not*I (GCGGCCGC) (53). For the *L. lactis* subsp. *cremoris* MG1363 chromosome, these endonucleases yielded 43, 24, and 3 bands, respectively, corresponding to fragment sizes of 3 to 610 kb (Table 1 and Fig. 1). The size of each fragment was estimated after PFGE separation under appropriate electrophoretic conditions (2.5-s pulse time for 11 h for fragments of <100 kb; 7.5-s pulse time for 13 h for fragments ranging from 100 to 300 kb; 15-s pulse time for fragments ranging from 250 to 500 kb; and 30-s pulse time for fragments from 450 to 700 kb) and by comparison with λ concatemers and a 1-kb ladder. Several gels were run under each electrophoretic condition, and the averages of the sizes of the restriction fragments are reported in Fig. 1. The genome size of MG1363 was thereby estimated as the total of the sizes of the restriction fragments to be 2,560 kb.

**Construction of physical map of MG1363 chromosome.** The physical map of the *L. lactis* subsp. *cremoris* MG1363 chromosome was constructed with *Apa*I, *Sma*I, and *Not*I as follows.

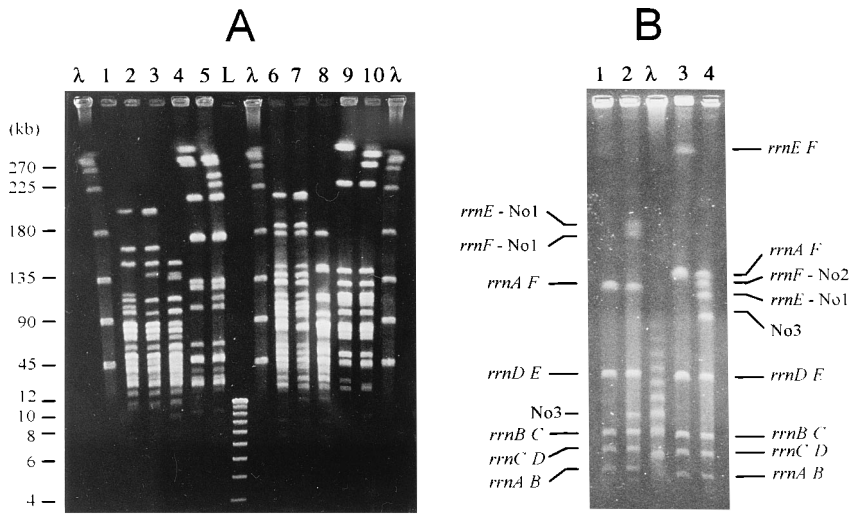


FIG. 1. PFGE restriction patterns of MG1363 and IL1403 chromosomes. (A) Typical combination of *Apa*I, *Sma*I, and *Not*I digestions. Lanes 1 to 5, MG1363 chromosome; 1, *Apa*I/*Not*I; 2, *Apa*I; 3, *Apa*I/*Sma*I; 4, *Sma*I; 5, *Sma*I/*Not*I. Lanes 6 to 10, IL1403 chromosome; 1, *Apa*I/*Not*I; 2, *Apa*I; 3, *Apa*I/*Sma*I; 4, *Sma*I; 5, *Sma*I/*Not*I. Lanes λ, λ DNA concatemers; lane L, 1-kb DNA ladder. Running conditions were 10 V/cm, 7.5 s of pulse time, for 13 h. (B) Digestion with I-*Ceu*I and *Not*I. Lanes: 1, MG1363 chromosome digested with I-*Ceu*I; 2, MG1363 digested with I-*Ceu*I and *Not*I; 3, IL1403 chromosome digested with I-*Ceu*I; 4, IL1403 digested with I-*Ceu*I and *Not*I; lane λ, λ DNA concatemers. Running conditions were 7.3 V/cm, 45 s of pulse time, for 24 h. The sizes of the I-*Ceu*I fragments of strain IL1403 are: Ce1, 1,480 kb; Ce2, 560 kb; Ce3, 240 kb; Ce4, 80 kb; Ce5, 45 kb; and Ce6, 18 kb.

**(i) Random insertion of *Apa*I, *Sma*I, and *Not*I restriction sites.** The integrative plasmid pRL1 integrates randomly into the chromosome of *Lactococcus* strains, creating additional *Apa*I, *Sma*I, and *Not*I restriction sites at the insertion point (50). Briefly, this integration uses the replicative transposition activity of the functional insertion sequence IS*S1*RS (39) and allows rapid unambiguous location of *Apa*I, *Sma*I, and *Not*I restriction sites close to the integration site by Southern analysis. The IS*S1*RS insertion sequence is also able to transpose efficiently in *Enterococcus faecalis* and *Streptococcus thermophilus* (59).

Fifty-one sites of integration of pRL1 were mapped precisely on the MG1363 chromosome (Table 2 and Fig. 2). Although some integration sites were clustered (the groups 8, 11, 17, and 53 and 2, 26, 37, 43, and 45 are each located within less than 50 kb), pRL1 integration sites seems to be distributed equally along the chromosome. Random insertion site distribution has already been observed in *L. lactis* subsp. *lactis* IL1403 (49). Analysis of only 35 integration sites (minimal set) allowed the unambiguous location of 51% of the *Apa*I and 54% of the *Sma*I restriction fragments.

**(ii) Completion of the physical map by indirect end labeling.** The remaining unmapped restriction sites were assigned on the MG1363 chromosome by the indirect end-labeling strategy (7), which allows the order of restriction fragments to be established relative to a fixed point. Indirect end-labeling experiments with 16 pRL1 derivative strains of MG1363 (Fig. 2 and Table 2) yielded a physical map of the MG1363 chromosome for these restriction sites except for the location of four *Apa*I restriction fragments (one of the three Ap24 fragments, one of the two Ap27 fragments, and the Ap30 and Ap31 fragments) and two *Sma*I fragments (Sm18 and Sm19).

**Construction of the genetic map.** The physical map was used as a guide for localizing genetic markers (Table 3) on the *L. lactis* subsp. *cremoris* MG1363 chromosome. Hybridization results with several of these probes helped improve the resolution of the restriction map. The genetic map of the *L. lactis* subsp. *cremoris* MG1363 chromosome was constructed by Southern hybridization analysis with homologous and heterologous probes. For some lactococcal genetic markers, accurate location and gene orientation on the chromosome could be obtained by homologous recombination using the integrative vector pRC1 (50).

**(i) Ribosomal operons (*rrn*).** The chromosome of lactococci contains six ribosomal operons (44, 49, 86). The complete sequence of one ribosomal operon of the *L. lactis* subsp. *lactis* IL1403 strain is available (10). The organization of this operon is similar to that described in the general model for eubacteria (i.e., 16S rRNA-23S rRNA-5S rRNA). Sequence analysis of this *rrn* operon also revealed the presence of unique recognition sites for three restriction endonucleases used for bacterial genome mapping: the *Apa*I and *Sma*I sites in the 16S rRNA gene (*rrs*), and the I-*Ceu*I site in the 23S rRNA gene (*rrl*) (56).

The six ribosomal operons were localized on the physical map of MG1363 strain by hybridization with probes specific for the 5′ end of the 16S rRNA gene and for the 3′ end of the 23S rRNA gene, derived from *Clostridium perfringens* (27). Each probe revealed six PFGE-separated fragments. Both the *Apa*I and *Sma*I hybridization patterns were different with the 16S and 23S probes, suggesting that each *rrn* operon contains one *Apa*I and one *Sma*I site. Ribosomal operon mapping allowed the location of the small restriction fragments Sm18 and Sm19 on the physical map. Sm18 hybridized only with the *rrl* probe, and Sm19 hybridized only with the *rrs* probe. Presumably, Sm18 maps between Sm11 and Sm2, and Sm19 maps between Sm13 and Sm10.

The position of each of the six rRNA operons on the chromosome, based on the *Apa*I and *Sma*I physical map, was also confirmed by using the intron-encoded endonuclease I-*Ceu*I (56). This enzyme, which recognizes a 26-bp sequence, cleaves specifically in the 23S rRNA gene of a wide range of organisms. The chromosomes of strains MG1363 and IL1403 were cleaved six times by I-*Ceu*I (Table 1 and Fig. 1), and the distance between each *rrn* operon, corresponding to each PFGE-separated fragment, was in agreement with their locations on the *Apa*I and *Sma*I physical map.

**(ii) Mobile genetic elements.** Four different insertion sequences in *L. lactis* have been described: IS*S1* (73), IS*904* (21),

TABLE 2. Analysis of pRL1 integration sites in the strain MG1363 chromosome[a]

| Strain | *Apa*I fragments | | *Sma*I fragments | | *Not*I fragments | |
|---|---|---|---|---|---|---|
| | Absent | Present (kb)[a] | Absent | Present (kb) | Absent | Present (kb) |
| CL56-1[b] | Ap1A | 200 + 6 | Sm8 | 65 + 43 | No1 | (1,905)[c] + 310 |
| CL56-2[b] | Ap4 | 55 + 95 | Sm1 | 425 + 190 | No2 | 65 + 170 |
| CL56-3 | Ap20 | 17 + 20 | Sm3 | 195 + 95 | No1 | 250 + (1,965) |
| CL56-4[b] | Ap13 | 11 + 55 | Sm7 | 50 + 80 | No1 | (1,635) + 580 |
| CL56-6 | Ap16 | 4 + 46 | Sm2 | 40 + 290 | No1 | (1,585) + 630 |
| CL56-7[b] | Ap5 | 70 + 50 | Sm2 | 130 + 200 | No1 | (1,730) + 485 |
| CL56-8 | Ap3 | 90 + 80 | Sm5A | 75 + 110 | No1 | (1,415) + 800 |
| CL56-10[b] | Ap6 | 85 + 20 | Sm3 | 115 + 175 | No1 | (1,980) + 235 |
| CL56-11 | Ap3 | 155 + 10 | Sm5A | 25 + 160 | No1 | (1,365) + 850 |
| CL56-12 | Ap10A | 45 + 35 | Sm5B | 20 + 165 | No1 | 760 + (1,455) |
| CL56-13 | Ap10B | 55 + 25 | Sm1 | 165 + 450 | No1 | (2,115) + 100 |
| CL56-14 | Ap1A | 100 + 105 | Sm1 | 40 + 580 | No1 | (1,995) + 220 |
| CL56-15 | Ap1A | 50 + 160 | Sm1 | 90 + 530 | No1 | (2,050) + 165 |
| CL56-17[b] | Ap3 | 140 + 25 | Sm5A | 40 + 145 | No1 | (1,385) + 830 |
| CL56-18 | Ap1A | 175 + 35 | Sm1 | 490 + 115 | No1 | 135 + (2,080) |
| CL56-20[b] | Ap14A | 26 + 31 | Sm10 | 28 + 27 | No1 | 910 + (1,285) |
| CL56-21[b] | Ap6 | 8 + 95 | Sm3 | 180 + 115 | No1 | 235 + (1,980) |
| CL56-22 | Ap14A | 21 + 35 | Sm10 | 34 + 23 | No1 | (1,295) + 920 |
| CL56-23 | Ap6 | 29 + 75 | Sm3 | 160 + 135 | No1 | 215 + (2,000) |
| CL56-24[b] | Ap2 | 23 + 162 | Sm5B | 37 + 145 | No1 | (1,315) + 900 |
| CL56-25 | Ap10B | 55 + 25 | Sm6 | 55 + 80 | No1 | (1,760) + 455 |
| CL56-26 | Ap4 | 75 + 75 | Sm1 | 410 + 205 | No2 | 45 + 185 |
| CL56-27 | Ap7 | 40 + 50 | Sm1 | 300 + 320 | No3 | 47 + 75 |
| CL56-28 | Ap1B | 91 + 125 | Sm1 | 23 + 590 | No2 | 10 + 225 |
| CL56-29[b] | Ap14B | 48 + 7 | Sm8 | 75 + 35 | No1 | 350 + (1,865) |
| CL56-30[b] | Ap2 | 115 + 55 | Sm5B | 178 + 7 | No1 | 930 + (1,285) |
| CL56-34 | Ap10C | 55 + 25 | Sm1 | 415 + 200 | No1 | 60 + (2,155) |
| CL56-35 | Ap17 | 10 + 35 | Sm1 | 390 + 225 | No1 | 30 + (2,185) |
| CL56-36 | Ap8 | 74 + 11 | Sm9 | 35 + 30 | No1 | 720 + (1,495) |
| CL56-37 | Ap4 | 100 + 50 | Sm1 | 375 + 220 | No2 | 25 + 210 |
| CL56-38[b] | Ap10A | 6 + 75 | Sm9 | 28 + 35 | No1 | (1,490) + 725 |
| CL56-39 | Ap1A | 170 + 50 | Sm1 | 515 + 100 | No1 | 150 + (2,065) |
| CL56-40 | Ap12B | 54 + 18 | Sm2 | 55 + 275 | No1 | (1,615) + 600 |
| CL56-41 | Ap1B | 80 + 130 | Sm16 | 8 + 13 | No1 | 22 + (2,193) |
| CL56-42[b] | Ap1B | 20 + 190 | Sm3 | 35 + 265 | No1 | 85 + (2,130) |
| CL56-43 | Ap4 | 85 + 65 | Sm1 | 400 + 220 | No2 | 35 + 200 |
| CL56-45 | Ap4 | 55 + 100 | Sm1 | 230 + 360 | No2 | 210 + 25 |
| CL56-46 | Ap12A | 60 + 20 | Sm2 | 30 + 300 | No1 | 360 + (1,855) |
| CL56-47 | Ap12B | 55 + 15 | Sm2 | 60 + 260 | No1 | (1,605) + 610 |
| CL56-48 | Ap10C | 73 + 7 | Sm1 | 400 + 220 | No1 | 50 + (2,165) |
| CL56-49[b] | Ap10B | 65 + 20 | Sm6 | 55 + 80 | No1 | (1,765) + 450 |
| CL56-50 | Ap7 | 30 + 60 | Sm1 | 300 + 305 | No3 | 55 + 65 |
| CL56-51 | Ap2 | 125 + 55 | Sm4 | 160 + 60 | No1 | (1,235) + 980 |
| CL56-52 | Ap7 | 77 + 11 | Sm1 | 285 + 335 | No3 | 35 + 80 |
| CL56-53 | Ap3 | 35 + 130 | Sm5A | 130 + 55 | No1 | 850 + (1,365) |
| CL56-54[b] | Ap1B | 8 + 200 | Sm1 | 110 + 495 | No2 | 90 + 145 |
| CL56-55 | Ap7 | 15 + 75 | Sm1 | 325 + 290 | No3 | 70 + 50 |
| CL56-56 | Ap19 | 18 + 22 | Sm14 | 20 + 6 | No1 | (1,217) + 1,000 |
| CL56-57 | Ap21 | 17 + 12 | Sm4 | 115 + 110 | No1 | 1,060 + (1,155) |
| CL56-58 | Ap9 | 49 + 38 | Sm5B | 85 + 105 | No1 | 835 + (1,380) |
| CL56-59[b] | Ap12A | 61 + 22 | Sm2 | 35 + 295 | No1 | 370 + (1,845) |

[a] The second fragment of each pair gave a strong signal when it was hybridized with pRL1.
[b] Strain used for indirect end-labeling experiments.
[c] Parentheses indicate that the fragment size was deduced from the size of *Not*I fragment No1 (2,215 kb).

IS*905* (22), and IS*981* (72). We attempted to map two of these insertion elements on the chromosome of strain MG1363. The first was the IS*S1*RS from the lactose protease plasmid pUCL22 of *L. lactis* subsp. *lactis* Z270 (39), which is very similar to IS*S1*. Previous results indicate that there are two chromosomal copies of IS*S1* in strain MG1363 (20). However, hybridization experiments revealed only one *Apa*I (Ap10) and one *Sma*I (Sm6) fragment. It was possible that the two copies were localized on the same *Apa*I and *Sma*I fragment. Therefore, Southern analysis by conventional agarose electrophoresis with *Eco*RI, *Hin*dIII, *Pst*I, or *Hae*III endonuclease was performed, and only one fragment hybridized with the probe. These results strongly suggested that the MG1363 chromosome contains only one copy of IS*S1*. The second IS element mapped on the MG1363 chromosome was IS*1076* from plasmid pUCL22 (40), which is closely related to the IS*904* element. Eight PFGE-separated bands scored positive after *Apa*I or *Sma*I digestion and hybridization experiments with IS*1076* as the probe, indicating that at least eight copies were present on the chromosome.

TABLE 3. Genetic markers localized on the IL1403 and MG1363 maps

| Element[a] | Description[b] | Source (species or *L. lactis* strain[c]) | Reference |
|---|---|---|---|
| Recurrent sequences, mobile elements, and bacteriophages | | | |
| 5′ *rrnA* | 16S rDNA (*rrs* gene) | *Clostridium perfringens* | 27 |
| 3′ *rrnI* | 23S rDNA (*rrl* gene) | *Clostridium perfringens* | 27 |
| IS*S1*RS (iso-IS*S1*) | Insertion sequence | *L. lactis* subsp. *lactis* Z270 | 39 |
| IS*1076* (iso-IS*904*) | Insertion sequence | *L. lactis* subsp. *lactis* Z270 | 40 |
| Tn*5276* target site 1 | Nisin-sucrose transposon | *L. lactis* NIZO R5 | 75 |
| ϕT712 | Temperate bacteriophage | *L. lactis* subsp. *cremoris* NCDO 712 | 16 |
| Lactococcal genes | | | |
| *adk** | Adenylate kinase | MG1363 | 45 |
| *aldB** | Acetolactate decarboxylase | MG1363 and IL1403 | 11 |
| *als* | α-Acetolactate synthase | DSM 20384 | 62 |
| *amdA* | Amidase | MG1363 | 63 |
| *bglR* | Positive regulator | IL1403 | 4 |
| *cluA* | Sex factor aggregation | MG1363 | 35 |
| *dnaE** | DNA primase | ML3 | 1 |
| *dnaJ* | Heat shock response | NIZO R5 | 89 |
| *dnaK* | Heat shock response | MG1363 | 25 |
| *fpg** | Formamidopyridine-DNA-glycosylase | ML3 | 24 |
| *gap* | Glyceraldehyde-3-phosphate dehydrogenase | NIZO R5 | 19 |
| *gerC** | Germination of *B. subtilis* | C2 | 31 |
| *grpE* | Heat shock response | MG1363 | 25 |
| *hisCGDBHAFIE* | Histidine biosynthesis | NCDO 2118 | 17 |
| *hisRF** | Histidyl-tRNA synthetase | ML3 | 76 |
| *ilvDBNCA* | BCAA biosynthesis | NCDO 2118 | 33 |
| *infA** | Initiation factor 1 | MG1363 | 45 |
| *lcnCD* | Lactococcin biosynthesis | IL1403 | 46 |
| *ldh* | Lactate dehydrogenase | NIZO R5 | 19 |
| *leuABCD* | BCAA biosynthesis | NCDO 2118 | 33 |
| *mleR* | MLF activator | IL1403 | 77 |
| *mleS* | MLF enzyme (Mlep) | IL1403 | 18 |
| *oppDFBCA* | Oligopeptide transport system | SSL135 | 87 |
| *pepC* | Thiol aminopeptidase | AM2 | 9 |
| *pepN* | Lysine aminopeptidase | Wg2 | 83 |
| *pepO* | Endopeptidase | SSL135 | 87 |
| *pepT* | Tripeptidase | MG1363 | 67 |
| *pepX* | X-prolyl dipeptidyl aminopeptidase | ML3 | 69 |
| *pip* | Phage infection protein | C2 | 31 |
| *recA* | Homologous recombination | ML3 | 24 |
| *rplO** | Ribosomal protein L15 | MG1363 | 45 |
| *rpmJ** | Ribosomal protein B | MG1363 | 45 |
| *rpoD* | $\sigma^{43}$ factor of RNA polymerase | ML3 | 1, 26 |
| *rpsM** | Ribosomal protein S13 | MG1363 | 45 |
| *rrg** | Glucosyltransferase regulator | C2 | 31 |
| *secY* | Protein translocation | MG1363 | 45 |
| *sod* | Superoxide dismutase | MG1363 | 81 |
| *thyA* | Thymidylate synthase | NCDO 712 | 79 |
| *trpEGDCFBA* | Tryptophan biosynthesis | IL1403 | 3 |
| *trxB* | Thioredoxin reductase | NCDO 2118 | 76 |
| *usp45* | Secreted protein | MG1363 | 88 |
| *uvrC* | Excision nuclease | IL1403 | 32 |
| Heterologous genes | | | |
| *gyrA* | DNA gyrase (subunit A) | *Clostridium perfringens* | 7 |
| "*rec*-like" | DNA recombination | *Streptococcus thermophilus* | 66 |
| *rpoA* | α subunit of RNA polymerase | *Escherichia coli* | 7 |
| *rpsL* | Ribosomal protein S12 | *Escherichia coli* | 7 |
| *uncD* | β subunit of ATP synthase F1 | *Escherichia coli* | 91 |

[a] The names of the ORFs marked with an asterisk are based on computerized protein sequence similarity searches. The *hisCGDBHAFIE*, *leuABCD-ilvDBNCA-aldB*, and *trpEGDCFBA-bglR* operons were localized by using the *hisF*, *ilvD*, and *trpC* genes as probes, respectively. The genes *dnaE* and *rpoD* are clustered (*rpoD* operon) (1) and were localized by using *rpoD* as the probe. The genes *rplO*, *secY*, *adk*, *infA*, *rpmJ*, and *rpsM* are clustered (45) and were localized by using *secY* as the probe. The genes *fpg* and *recA* are clustered (24) and were localized by using *recA* as the probe. The genes *pip*, *gerC*, and *rrg* are clustered (31) and were localized by using *pip* as the probe. The genes *grpE* and *dnaK* are clustered (25) and were localized by using *dnaK* as the probe. The genes *oppDFBCA* and *pepO* are clustered (87) and were localized by using *pepO* as the probe.

[b] BCAA, branched-chain amino acids; MLF, malolactic fermentation.

[c] Strains MG1363, ML3, C2, AM2, Wg2, and NCDO 712 are *L. lactis* subsp. *cremoris*; NIZO R5 is *L. lactis*; all other strains are *L. lactis* subsp. *lactis.*

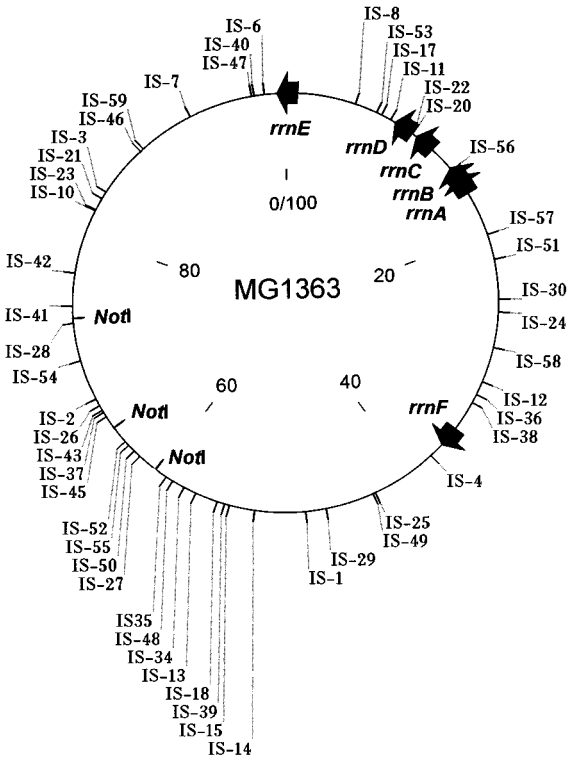


FIG. 2. Chromosomal locations of pRL1 integration sites (IS). IS-*x* corresponds to CL56-*x* in Table 2, where *x* is a number from 1 to 59. In order to facilitate locating the integration sites, the positions of the three *Not*I sites and of the positions of the six ribosomal operons are indicated.

Many industrial strains of *L. lactis* produce antimicrobial peptides, and the genes responsible for the production of and resistance to these compounds have been found on conjugative transposons (21, 75). Rauch et al. demonstrated that the nisin-sucrose transposon Tn*5276* was able to integrate at various locations in the MG1363 chromosome but showed a preference for insertion into a single target site, designated site 1. Preliminary PFGE analysis showed that Tn*5276* insertion site 1 was located on 200-kb *Sma*I and on 12-kb *Eag*I fragments (75). By using a 4.3-kb *Hin*dIII chromosomal DNA fragment of strain MG1614 containing site 1 as the probe, it was possible to assign integration site 1 to fragments Ap2 and Sm5B.

**(iii) Integration sites of temperate bacteriophages.** The location of prophage DNA on the bacterial chromosome can be of interest for the study of interactions at the DNA level between bacteriophages and the host cell. Various studies show that temperate bacteriophages are responsible for the PFGE restriction fragment length polymorphism of the *Staphylococcus aureus* (82), *Clostridium perfringens* (8), and *Salmonella typhimurium* (95) chromosomes.

The attachment site (*attB*) of the lactococcal temperate phage TP901.1 was precisely located on the MG1363 and IL1403 chromosomes. The integrative plasmid pBC143 (13) contains the TP901.1 *attP* site, the integrase gene, and single *Apa*I and *Sma*I restriction sites. When introduced into lactococcal cells, it integrates into the chromosome via specific recombination. The parental restriction fragment (*Apa*I or *Sma*I) disappears and is replaced by two new fragments, which can be revealed by hybridization with the pBC143 probe.

The putative attachment site of the temperate bacteriophage φT712 was located by using a DNA fragment of the phage as the probe (data not shown).

**(iv) Lactococcal and heterologous genetic markers.** Thirty-three cloned lactococcal or heterologous genetic markers were located on the MG1363 physical map by Southern hybridization under high-stringency (homologous genes) or low-stringency (heterologous genes) conditions with PFGE-separated restriction fragments after combinations of single and double digestions with *Apa*I, *Sma*I, and *Not*I (Fig. 1A). Some lactococcal genes were more precisely located on the chromosome by another procedure. The positions of the lactococcal *ilvD*, *thyA*, *rpoD*, *ldh*, *pepX*, and *pepC* genes were determined by using the integrative vector pRC1. When a lactococcal gene is introduced into plasmid pRC1, the resulting plasmid can integrate into the chromosome via homologous recombination, and this insertion allows the accurate mapping of the gene (50). In addition, if the transcription direction of the gene is known, the gene orientation (5′ → 3′) on the chromosome can be deduced from hybridization data. The *recA* gene was localized by using an MG1363 *recA* strain (23) constructed by insertional mutagenesis with a thermosensitive integrative plasmid (60). As this mutagenic event creates new *Apa*I, *Sma*I, and *Not*I sites at the insertion point, the strategy described above was used to locate the *recA* gene and to deduce its orientation of transcription on the MG1363 chromosome. The *cluA* gene contains an *Apa*I site (32). This gene was thus precisely localized on the MG1363 physical map by classical Southern hybridization, using probes specific for the 5′ end and for the 3′ end of the *cluA* gene, and confirmed the location of the Ap26 fragment between the Ap1B and Ap4 fragments.

As many mapped genes were either organized in operons (*ilv*, *his*, and *trp* operons) or clustered with other characterized open reading frames (ORFs) (*secY*, *rpoD*, and *pip* genes), the 33 probes used in this study allowed a total of 77 chromosomal genetic markers to be mapped.

A combined physical and genetic map of the *L. lactis* subsp. *lactis* MG1363 chromosome is shown in Fig. 3 and is compared with that of *L. lactis* subsp. *lactis* IL1403 in Fig. 4. Overall, the gene organization is conserved. However, strain MG1363 presents a large inversion of one half of the genome in the region containing the rRNA operons.

One interesting feature of the *Lactococcus* chromosome is the location of genes encoding the subunits of RNA polymerase. When the *rpoA* gene of *E. coli* was used as the probe, two *Apa*I and *Sma*I fragments were revealed with the same intensity, suggesting the presence of two copies of the *rpoA* gene in MG1363. IL1403 also contains two copies of the *rpoA* gene (49), although one copy hybridized weakly with the probe. One copy (arbitrarily named *rpoA*-1) mapped between the *rrnA* and *rrnF* operons, and the second mapped near the cluster of genes *secY-adk-infA-rpmJ-rpsM*, as found on the chromosomes of *E. coli* (2) and *Bacillus subtilis* (71). The precise location of the *rpoD* gene was determined by homologous recombination and this gave further information on the gene organization near this ORF. It was previously observed that the lactococcal *rpoD* gene was associated with the *dnaG* gene in an operon (1). In *E. coli* and *B. subtilis*, the *rpoD* gene is located in an operon called the macromolecular synthesis (MMS) operon (90). This operon contains the gene coding for DNA primase (*dnaG* in *E. coli* and *dnaE* in *B. subtilis*) and either a gene coding for S21 ribosomal protein (*rpsU*) in *E. coli* or a gene of unknown function (P23) in *B. subtilis*. In contrast to the position of the "*rpoD* operon" in *E. coli* and *B. subtilis*, which is very distant from any *rrn* operon, the *rpoD* operon is very closely associated with the *rrnF* operon (less than 3 kb) in the MG1363 chromosome.

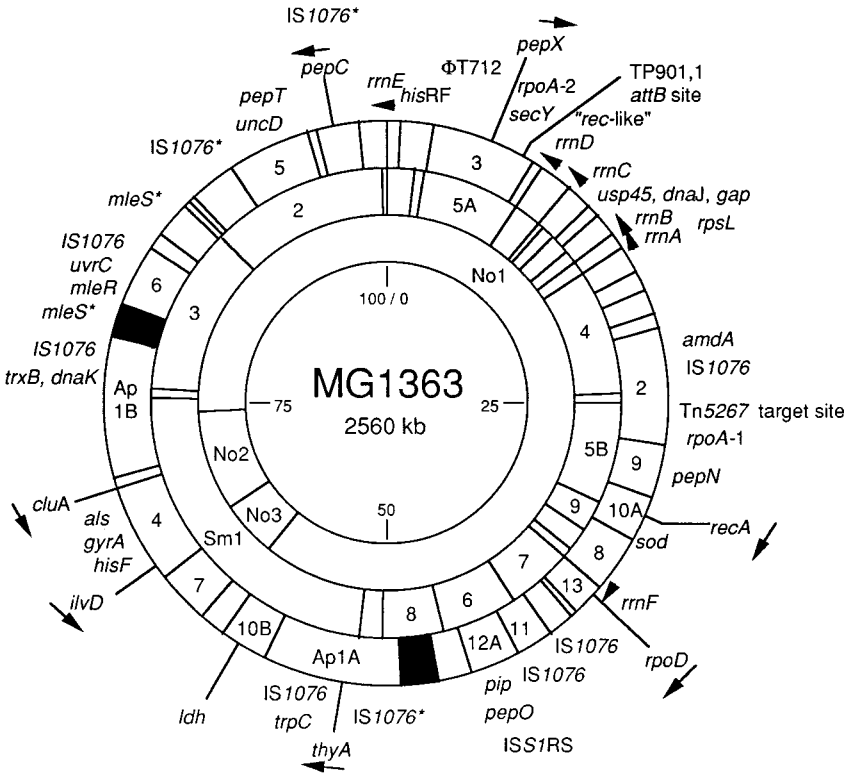


FIG. 3. Physical and genetic map of the chromosome of *L. lactis* subsp. *cremoris* MG1363. Restriction fragment number is indicated in some cases. For easier genome comparison, the origin of the map (0/100) is arbitrarily located at the *rrnE* operon. The *Apa*I restriction fragment order is Ap24A-Ap14A-Ap3-Ap25-Ap14B-Ap18-Ap21-Ap19A-Ap23-Ap15-Ap24B-Ap19B-Ap22-Ap2-Ap9-Ap10A-Ap8-Ap13-Ap31-Ap19C-Ap11-Ap10B-Ap14C-unmapped region-Ap1A-Ap10C-Ap17-Ap7-Ap4-Ap26-Ap1B-unmapped region-Ap6-Ap20-Ap14D-Ap27B-Ap29-Ap12A-Ap5-Ap28-Ap12B-Ap16. The *Sma*I restriction fragment order is Sm11A-Sm15-Sm5A-Sm10-Sm19-Sm13-Sm12-Sm14A-Sm14B-Sm4-Sm14C-Sm5B-Sm9-Sm11B-Sm17-Sm7-Sm6-Sm8-Sm11C-Sm1-Sm16-Sm3-Sm2-Sm18. The scale is arbitrary, and units are a percentage of the total size. Asterisks indicate the ambiguous locations of probes. Arrows indicate the 5′ → 3′ orientation of the gene. Solid boxes correspond to unmapped *Apa*I fragments.

Another characteristic of the *Lactococcus* genome is the chromosomal location of the heat shock gene *dnaK* relative to the position of the *dnaJ* gene. In many organisms, *dnaK* is associated with the heat shock *dnaJ* gene, and in some cases with the *grpE* gene, in an operon. Sequence analysis of the MG1363 *dnaK* region (25) revealed that *dnaJ* was not part of the *grpE-dnaK* operon, and indeed, the *grpE-dnaK* operon is far from the *dnaJ* gene (900 kb for MG1363 and 1,050 kb for IL1403).

## DISCUSSION

This paper reports the first construction of a combined physical and genetic map of an *L. lactis* subsp. *cremoris* chromosome. The strategy used combined two complementary techniques: the generation of new rare restriction sites by random insertion of the integrative plasmid pRL1, and indirect end labeling. An important task in the construction of the physical map of a genome for more than one enzyme is to determine the exact locations of restriction sites for the second enzyme in the restriction fragments of the first enzyme. This can be done in a simple and reliable way by using pRL1, which minimizes the risk of mistakes in the restriction map, especially for fragments of similar size, because it allows the unambiguous pairing of one *Apa*I and one *Sma*I fragment and their precise locations with respect to the *Not*I restriction fragments.

Although only 35 integration sites (called the minimal set) were sufficient for matching various *Apa*I and *Sma*I fragments, the 16 remaining insertion sites analyzed were not unproductive for the construction of the physical map. These redundant sites can be useful for other mapping techniques, such as indirect end labeling, or for other analyses of the chromosome, such as chromosomal DNA transfer during conjugation (30) and fine analysis of genome rearrangements between closely related strains (unpublished data). The second mapping method, indirect end labeling, is a very powerful tool, as it produces internally consistent maps in a fast and nonrandom manner. However, when restriction fragments of similar sizes are too numerous, this technique does not allow unequivocal location of all the fragments. This was particularly the case for the construction of the *Apa*I restriction map of the MG1363 chromosome, in which two regions, each covering about 50 kb, could not be mapped.

The size of the MG1363 chromosome, estimated by independently summing the sizes of the *Apa*I and *Sma*I restriction fragments, is about 2,500 kb. This value is consistent with previous genome size determinations for other *L. lactis* strains (53, 84) and is similar to that of the genomes of other lactic acid bacteria, such as *Lactobacillus* spp., *Leuconostoc* spp., and *Streptococcus thermophilus* (for a review, see reference 51), and various *Streptococcus* species (28, 37). A recent review (14) proposed classifying bacterial genome sizes into groups: 0.6 to 1.5 megabases (Mb) (group 1), 1.5 to 3 Mb (group 2), 3 to 4.5 Mb (group 3), and >4.5 Mb (group 4). The genome size of *L. lactis* strains and other lactic acid bacteria falls into group 2, which includes pathogenic bacteria, such as *Haemophilus* spp.,

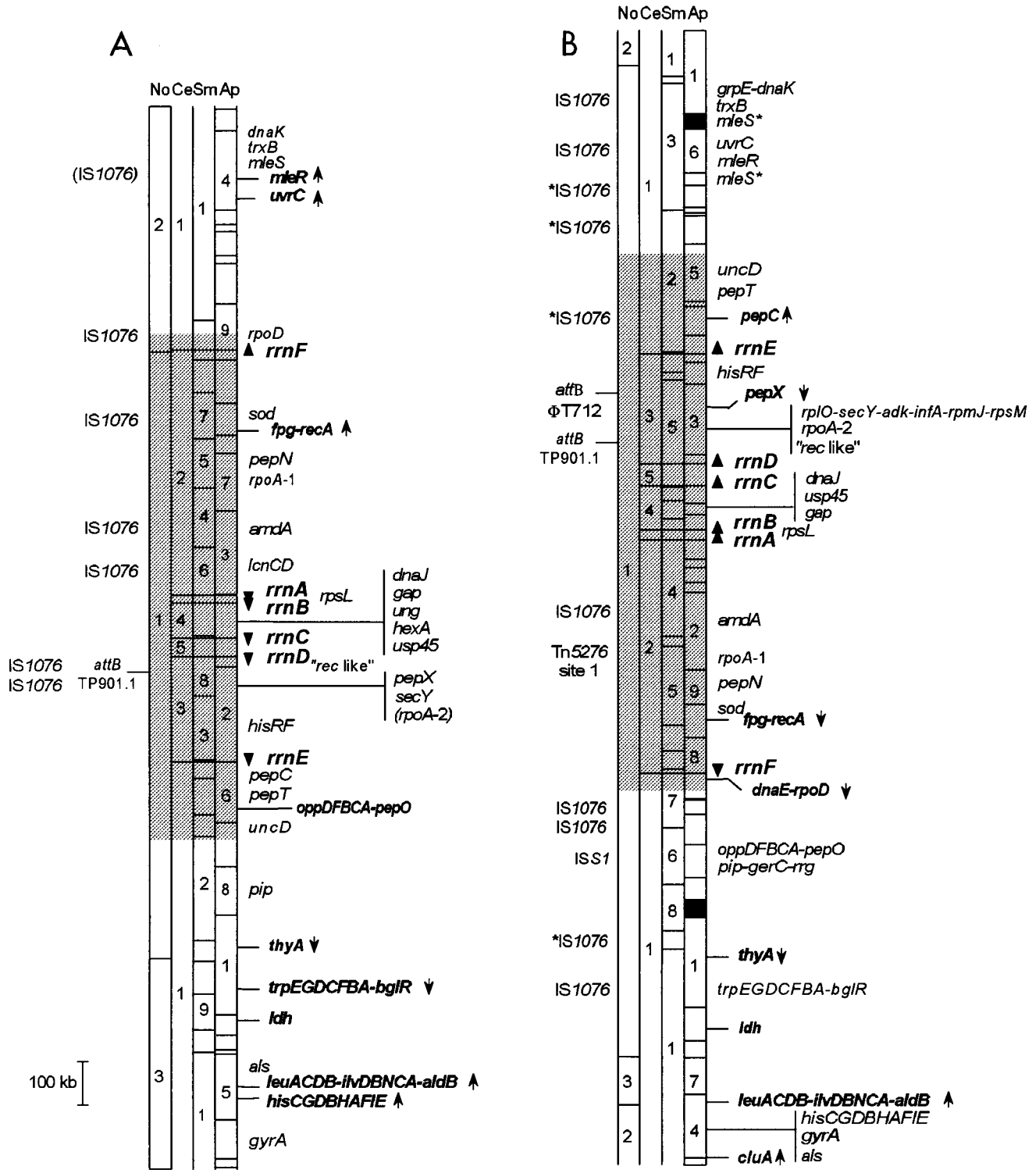


FIG. 4. Comparison of the chromosome maps of (A) *L. lactis* subsp. *lactis* IL1403 and (B) *L. lactis* subsp. *cremoris* MG1363. Genes that were precisely located on the physical maps are shown in boldface. Arrows indicate the 5′ → 3′ orientation of the gene. Parentheses indicate genes which hybridized weakly with the corresponding fragment. Shaded regions indicate the genome inversion between the two strains. Solid boxes correspond to unmapped *Apa*I fragments. Asterisks indicate the ambiguous locations of the probes. Abbreviations: Ap, *Apa*I; Ce, I-*Ceu*I; No, *Not*I; Sm, *Sma*I.

*Neisseria gonorrhoeae*, *Campylobacter* spp., and *Brucella melitentis* (gram-negative bacteria), and *Enterococcus faecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus* (gram-positive bacteria).

The physical map of the *L. lactis* subsp. *cremoris* MG1363 chromosome was compared with the previously published map of the two *L. lactis* subsp. *lactis* strains IL1403 (Fig. 4) and DL11. In contrast to the good correspondence observed along 60% of the DL11 and IL1403 physical maps (49), the physical map of the MG1363 chromosome is very different, as seen

when comparing the *Apa*I and *Sma*I restriction profile with those of these two reference strains (Fig. 1A). These polymorphisms could be explained by the nucleotide sequence divergence between some coding regions of the subspecies *lactis* and *cremoris*, which has been estimated to be between 20 and 30% (34). This difference is of the same order of magnitude as that observed between *E. coli* and *S. typhimurium*, which separated about 100 millions years ago (70). We suggest that for strains belonging to the same subspecies (i.e., *lactis* for the DL11 and IL1403 strains), the restriction polymorphism observed was due mainly to genome rearrangements rather than point mutations. For strains of different subspecies (i.e., *cremoris* and *lactis* for MG1363 and IL1403), this polymorphism may be caused by sequence divergence as well as genome rearrangements. A recent study (36) demonstrated that restriction polymorphism between different isolates of *E. faecalis* was much more frequently due to DNA rearrangements than to point mutations.

The construction of an accurate genetic map is valuable because it provides access to the genome organization and gives better clues to genome rearrangements than does a physical map. The backbone of a bacterial chromosome can be defined with a limited set of key housekeeping genes, including ribosomal operons (*rrn*). The strain MG1363 chromosome contains six *rrn* operons; five are clustered into 15% of the chromosome and are transcribed in the same direction, and the sixth (arbitrarily named *rrnF*) is transcribed in the opposite direction. The six operons are located on 36% of the genome and organized in the same manner as the *rrn* operons of *L. lactis* subsp. *lactis* IL1403 and DL11. This unequal distribution is analogous to that found in most prokaryotes, in which *rrn* operons are located on 37 to 52% of the chromosome and generally found to be within two groups that are transcribed divergently, away from the origin of replication (*oriC*). If the position of *oriC* with respect to the *rrn* operons is conserved in the *Lactococcus* genome, the chromosomal replication origin should be located near the *rrnA* operon in MG1363 and IL1403. Moreover, the lactococcal genes that were precisely mapped were transcribed in the same direction as the *rrn* operons in both strains MG1363 and IL1403 (Fig. 4) except for the *pepX* gene on the MG1363 chromosome. These observations are in agreement with those for *E. coli* and *B. subtilis*, in which most genes are transcribed in the same direction as replication occurs (6, 96). The I-*Ceu*I endonuclease, which cleaves a sequence of the *rrl* gene, which is strongly conserved among bacteria and the organelles of many eukaryotes (56), was used to compare directly the number and location of ribosomal operons in MG1363 and IL1403 (Fig. 1B). The position of each *rrn* in the two strains is conserved, since there was little or no size difference in the I-*Ceu*I fragments, especially fragments Ce5, Ce4, and Ce3.

Knowledge of the positions of mobile genetic elements on the bacterial chromosome contributes to our understanding of chromosomal dynamics. In *E. coli*, IS elements are involved in genome rearrangements such as DNA amplifications (68), small deletions (5), and large chromosomal inversions (47). It has been proposed that during protoplasts fusion in *L. lactis* subsp. *cremoris*, insertion sequences are also implicated in rearrangement of genetic material (93). The presence of only one chromosomal copy of the IS*S1* insertion sequence in MG1363, together with its absence from the IL1403 chromosome, strongly suggests that IS*S1* is not primarily responsible for genomic rearrangements in these two *Lactococcus* strains. The number and location of the IS*1076* elements are not conserved between the MG1363 and IL1403 chromosomes, since the nine copies are located in one half of the IL1403 chromosome, whereas the seven copies present in the MG1363 strain are distributed all along the chromosome.

Comparison of the genetic maps of the two strains provides information on genome dynamics in *L. lactis* strains. Despite a good correlation between the gene orders on the two chromosomes, they differ by the presence of a large chromosomal inversion between IL1403 and MG1363 that covers about 50% of the genome and contains the six *rrn* operons (Fig. 4). The borders of this inversion are located near the *uncD* and *rpoD* genes. However, the position of the *oppDFBCA-pepO* gene cluster, located close to one side of the inversion, is not conserved between the IL1403 and MG1363 chromosomes, probably indicating that the genome rearrangement observed between the two strains is more complex than a single inversion event. Interestingly, close analysis of the integration sites of pRL1 in the MG1363 (Fig. 2) and IL1403 (49) chromosomes indicates that the borders of the inversion are located in regions that are relatively devoid of integration events, between IS-4 and IS-25 or between IS-59 and IS-47 on the MG1363 map.

Genome comparison between different species of bacteria reveals that gene organization is relatively conserved even for organisms which diverged 100 million years ago, as for *E. coli* and *S. typhimurium*, suggesting that selective forces or mechanistic restrictions act to preserve gene order. In addition, among the different types of genome rearrangements (i.e., amplification, deletions, and inversions), naturally occurring large inversions are rarely found in bacterial chromosomes. At the species level, two chromosomal inversions including the replication terminus have been reported: the well-characterized inversion between *E. coli* and *S. typhimurium* (78), and an inversion between *Salmonella enteritidis* and *S. typhimurium* (55). At the intraspecies level, only a few inversions between *E. coli* laboratory strains have been described, and all involved recombination between two similar, oppositely oriented sequences, such as the *rrn* operons for strain W3110 (38) and IS elements for strains 1485IN (47), BHB2600 (15), and LN1091 (57). Recently, genome map comparisons based on gene location revealed the presence of a chromosomal inversion between two serovars of *Leptospira interrogans* (97) and on the chromosome of different strains of *Mycoplasma hominis* (48). In *L. lactis*, the chromosomal inversion between IL1403 and MG1363 does not involve homologous recombination between two *rrn* operons, since all are located inside the inverted region or between two IS*1076* insertion sequences, since no copy of this element could be found close to the borders of the inversion.

The presence of this large chromosomal inversion raises the question of the degree of plasticity in the species *L. lactis*. The frequency inversion has to be evaluated: is it of common occurrence and observable even in isogenic strains, or is it the result of an ancestral event that took place before the divergence into the two subspecies *lactis* and *cremoris*?

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